Leukemia and Lymphoma, 2002 Vol. 43 (7), pp. 1367–1373

B Lymphocyte Stimulator (BLyS): A Therapeutic Trichotomy for the Treatment of B Lymphocyte Diseases

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(Received 20 December 2001)

B Lymphocyte Stimulator (BLyS™ protein) is a member of the tumor necrosis factor (TNF) family of ligands and functions as an essential *in vivo* regulator of B lymphocyte homeostasis. As such, changes in systemic BlyS protein expression caused by disruption of the gene encoding BLyS or administration of neutralizing soluble receptors have resulted in profound losses in mature B cell numbers and impaired humoral immunity. A similar phenotype has been observed in A/WySnJ mice that express a truncated BLyS receptor and are thus defective in BLyS signal transduction. In contrast, overexpression of BLyS protein in BLyS-transgenic mice results in B cell hyperplasia, hypergammaglobulinemia, and development of autoimmune-like disease. The ability of BLyS to regulate both the size and repertoire of the peripheral B cell compartment raises the possibility that BLyS and antagonists thereof may form the basis of a therapeutic trichotomy. As an agonist, BLyS protein may enhance humoral immunity in congenital or acquired immunodeficiencies such as those resulting from viral infection or cancer therapy. BLyS-specific antagonists (antibodies or soluble receptors) that inhibit the biological activity of BLyS may be effective therapies for those autoimmune diseases characterized by polyclonal hypergammaglobulinemia and elevated autoantibody titers. Finally, the specificity of BLyS for B-lineage cells raises the possibility that BLyS may be used as a targeting vehicle for delivery of a cytotoxic or cytolytic signal to neoplastic B-lineage cells expressing one or more of the three known BLyS receptors. This review discusses the therapeutic potential of BLyS in the context of BLyS structure, function and receptor specificity.

Keywords: BLyS; B cells; Proliferation; Autoimmunity; Apoptosis; Tumors

INTRODUCTION

B lymphocyte development is a controlled process of differentiation that begins in the bone marrow, where committed B-lineage precursor cells undergo immunoglobulin (Ig) heavy and light chain gene rearrangements. If these rearrangements are successful, the immature B cell gains antigen responsiveness through expression of the B cell receptor complex (BCR) on its cell surface. At the same time, other receptors and ligands are expressed, enabling the immature B cell to emigrate to lymphoid tissues and interact with its new peripheral microenvironment [1–3]. However, of the 10–15 million immature B cells produced each day by a healthy mouse, only about 3% actually further differentiate into mature, long-lived B cells, suggesting the existence of strong selective and homeostatic mechanisms controlling the size and repertoire of the mature B cell compartment [4–6]. The mechanisms controlling the transition from the immature bone marrow B cell to the more mature peripheral B cell are not fully understood but clearly involve extensive negative selection to eliminate autoreactive BCR-expressing cells and positive selection to define an effective antigen-specific repertoire [7–12]. The composition of the peripheral B cell pool is also controlled by BCR-independent mechanisms through costimulatory signals such as CD40/CD40L [13], CD45 [14], and the recently described cytokine BLyS (BAFF, TALL-1, zTNF4, TNFS20, THANK) [15–19]. Unlike other costimulatory signals, BLyS participates in the regulation of both the size and repertoire of the peripheral B cell pool, suggesting that BLyS, its receptors, and antagonists thereof are intriguing therapeutics for the treatment of B cell diseases. This review will summarize the biology of BLyS, its multiple receptors, and the potential use of BLyS as the basis of a therapeutic trichotomy for the treatment

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ISSN 1042-8194 print/ISSN 1029-2403 online © 2002 Taylor & Francis Ltd
DOI: 10.1080/10428190290033297

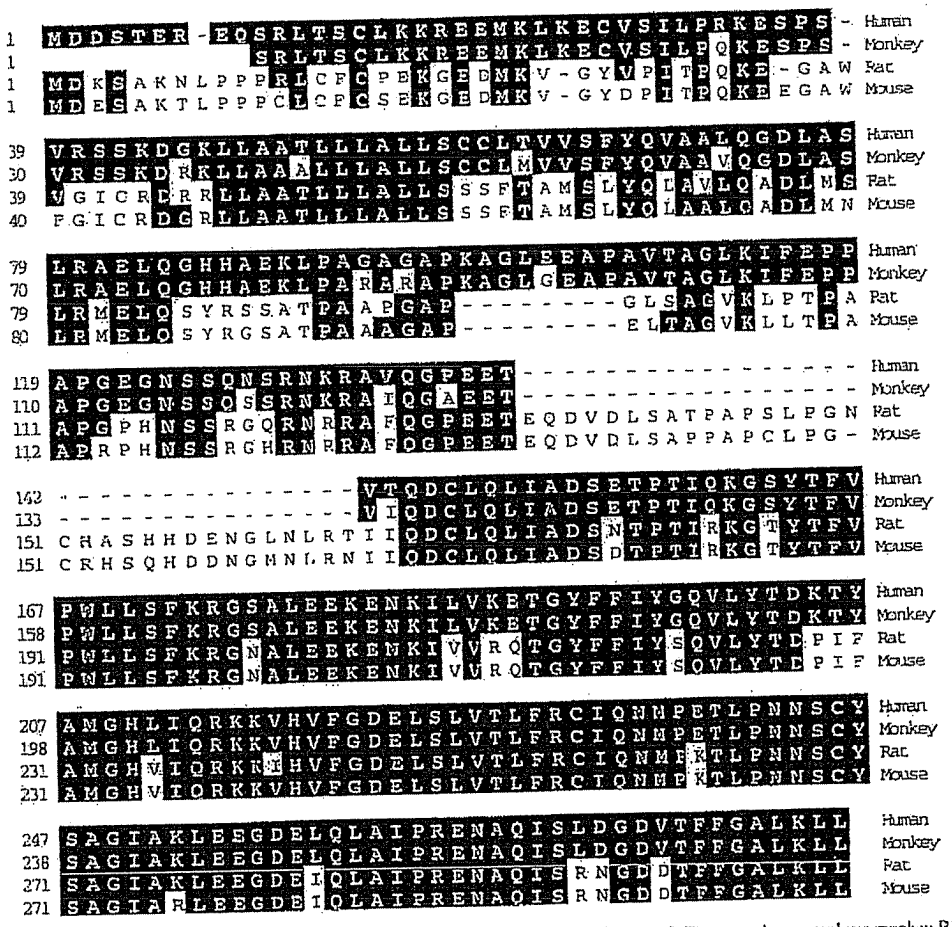

FIGURE 1 Clustal alignment of the amino acid sequences of human, monkey, rat and murine BLyS. The rat and cynomolgus monkey BLyS sequences were obtained through degenerate PCR performed on spleen cDNA using probes based on the human and murine sequences. The monkey sequence provided lacks approximately 10 N-terminal amino acids. An additional variant of the rat BLyS cDNA (lacking amino acids 133–165) was also identified (Feng and Moore, unpublished). Amino acids with a black background match the human BLyS sequence exactly.

TABLE 1 BLyS expression and binding to human cell lines

Cell line	Cellular morphology	BLyS expression	BLyS binding
Daudi	Burkitt's lymphoma	–	–
Namalwa	Burkitt's lymphoma	–	+
Raji	Burkitt's lymphoma	–	+
IM-9	Multiple myeloma	.	+
RPMI-8226	Multiple myeloma	.	+
Reh	Acute lymphocytic leukemia	–	.
Jurkat	Acute T cell leukemia	–	–
Sup-T13	Acute T cell leukemia	–	
K562	Chronic myelogenous leukemia	+	
HL-60	Acute promyelocytic leukemia	+	–
THP-1	Acute monocytic leukemia	+	–
U937	Histiocytic lymphoma	+	–
HCT116	Colon carcinoma	–	–
HT29	Colon carcinoma	–	–
CACO-2	Colon adenocarcinoma	–	–
COLO-201	Colon adenocarcinoma	–	–
MDA-MB-231	Breast adenocarcinoma	–	–
SCaBER	Bladder squamous carcinoma	–	–
A498	Kidney carcinoma	.	–
HST766T	Pancreas carcinoma	.	

Flow cytometric analysis was conducted using a biotinylated mAb BLyS-specific (BLyS expression) or biotinylated BLyS recombinant protein (BLyS binding) followed by phycoerythrin-conjugated streptavidin.

of immunodeficiencies, autoimmunity and neoplastic B cell diseases.

BLYS

The BLyS cDNA sequence was first identified through high-throughput cDNA sequencing of a human neutrophil-derived cDNA library on the basis of its sequence homology to other tumor necrosis factor (TNF) like molecules, most notably APRIL [15]. Structural analysis of the 285-amino-acid BLyS protein sequence predicts a type II transmembrane protein with a carboxy-terminal extracellular domain. Comparison of human, murine, rat and monkey BLyS sequences (Fig. 1) demonstrates strong conservation within the receptor-binding carboxy-terminal region with more sequence variation observed in the N-terminal region. Compared to the human and monkey BLyS sequences, murine BLyS contains an extra insertion between the transmembrane region and the first predicted β-sheet [15,16]. The BLyS gene has been mapped to human chromosome 13q34 [15,16]. Of the known TNF ligands, only RANKL has been located on this chromosome, although quite distant from BLyS.

BLyS is encoded by a single 2.6 kb mRNA highly expressed in peripheral blood mononuclear cells, and also found in spleen, lymph nodes, and bone marrow [15–19]. Although Schneider *et al.* have detected BLyS mRNA in T cells [16], the protein is constitutively expressed on the cellular membrane of tumor cells of myeloid lineage (Table I), monocytes, macrophages and dendritic cells, as shown by flow cytometric analysis [15,18,20]. Among primary cells, BLyS expression is upregulated by IFN-γ [20] and downregulated by phorbol myristate acetate/ionomycin and by LPS [17,18]. Primary myeloid cells and tumor cells of monocytic origin produce also a soluble form of the protein [20], thus indicating that BLyS, like other ligands of the TNF family [21], is both a membrane-bound and secreted protein. Mutations in the polybasic sequence immediately preceding amino acids K132 and R133 prevented the release of soluble BLyS [20]. This finding is consistent with the hypothesis that a protease, possibly a furin, is responsible for the cleavage of the membrane-bound protein [16].

Recombinant human BLyS has been produced in prokaryotic and eukaryotic expression systems [15,16,18,19] as a soluble protein of 17 kD, with a N-terminal sequence beginning with Alanine-134. Gel filtration analysis showed that the recombinant protein assembles into a trimer [16], similarly to the quaternary structure of other TNF family members.

BLYS RECEPTORS

To date, three cellular receptors for BLyS have been identified: transmembrane activator and CAML-interactor (TACI), B cell maturation antigen (BCMA), and BAFF-receptor/BLyS receptor 3 (BAFF-R or BR3) [22–30]. Unlike most members of the TNF family of receptors that are type I transmembrane proteins [31], TACI, BCMA and BAFF-R are type III transmembrane receptors. Each has the expected cysteine-rich repeats but the most distantly related member, BAFF-R, has only four cysteines in its ligand binding domain compared to the six cysteine residues found in TACI and BCMA.

Early analyses utilizing FLAG-tagged and biotinylated forms of BLyS identified BLyS-binding proteins on the surface of normal and neoplastic B-lineage cells ([15,16], and Table I). Subsequent analyses using a ^{125}I-BLyS probe demonstrated that BLyS binds with high affinity to human tonsillar B cells ($K_d = 0.1$ nM) and the number of binding sites was determined to be approximately 2600 per cell [32].

TACI and BCMA were the first two BLyS receptors identified [22–28]. TACI was originally reported to interact with calcium-modulator and cyclophilin ligand (CAML) involved in the signaling events underlying lymphocyte activation [33]. TACI, which contains two repeated cysteine-rich regions that are 50% identical to one another, is expressed on B cells and activated T cells. This pattern of expression accounts for the activity observed following BLyS treatment in activated T-cell populations [18], as well as the blocking effect of the soluble receptor on T-cell activation [34]. BCMA was first described as a B-cell maturation antigen expressed primarily in immune organs and mature B cell lines [35]. It contains only one extracellular six-cysteine-rich motif, corresponding to a variant motif present in the fourth repeat of the TNFR1 molecule. Both TACI and BCMA signal as classic TNF-like receptors, through activation of NF-κB and AP-1 via the TRAF family of adaptors [24–26,28,36]. TACI and BCMA are also receptors for APRIL, another member of the TNF family. BCMA binds preferentially to APRIL; the functional outcome of this interaction is still unclear, although it has been reported to have a potential role in induction of tumorigenesis [37].

To define the role of these receptors in B cell development, several groups have generated mice lacking TACI or BCMA. Surprisingly, TACI-deficient mice [38,39] had increased B cell accumulation and splenomegaly resulting from increased cellularity of B cell follicles and marginal zones. All the B cell types were present in increased numbers, including mature and immature transitional 1 (T1) B cells. The B cells hyperproliferated and produced more IgM, IgG and IgE in response to various stimuli *in vitro*. Moreover, challenge with T-cell-dependent antigens resulted in enhanced antigen-specific antibody response, although responses to T-cell-independent type II antigens were almost completely abolished. From the analysis of the knockout mice, it appeared that TACI is mainly a negative regulator of B-cell survival and may have a role in maintaining homeostasis. BCMA-deficient mice, on the other end, had no peculiar phenotype or B-cell deficiency

[40]. All the B-cell types, from precursors to mature populations, were present. Taken together, these studies indicated that additional receptor(s) mediate the mitogenic activity of BLyS.

The third receptor BAFF-R, was finally identified by Thompson *et al.* [29] and by Yan *et al.* [30]. BAFF-R is characterized by one cysteine-rich domain containing only four cysteines, making it the smallest molecule of the TNF receptor family. This single motif is similar to the C2 repeat of BCMA and TACI. BAFF-R mRNA is found in secondary lymphoid organs and it is absent in fetal liver or bone marrow and, as for BCMA, its expression is restricted to B cells. The transcript is highly expressed by resting B cells and, in contrast to TACI, appears to be downregulated upon activation. BAFF-R interacts only with BLyS and does not bind any other TNF ligand, including APRIL. Importantly, it was found that the immunodeficient A/WySnJ mice have a mutation in the gene coding for the intracellular domain of BAFF-R, so that the signaling through the receptor is interrupted. The A/WySnJ mice exhibit a B-cell phenotype similar to that of BLyS deficient animals, with loss of late transitional 2 (T2) B cells, but normal bone marrow and early T1 subsets and peritoneal B-1 cells.

BLYS AS A STIMULATOR OF HUMORAL IMMUNITY

The first indication of the key role of BLyS in promoting humoral immunity came from *in vitro* experiments using recombinant protein in standard B cell co-stimulation assays [15,16]. BLyS increased the proliferation and the antibody production of human B cells costimulated by anti-Ig M or by formalin-fixed *Staphylococcus aureus Cowan I* (SAC). Subsequent *in vitro* studies by Do *et al.* have demonstrated that BLyS affects the humoral responses to T-cell-dependent and -independent antigens by reducing apoptosis in B cells [41]. The increase in the survival of naïve resting B cells as well as CD40-activated cells was found to correlate with changes in the ratio of Bcl-2 family proteins, with reduction in expression of the pro-apoptotic Bak and increase of anti-apoptotic Bcl-2 and Bcl-x_L. Moreover, Batten *et al.* reported that BLyS increases the survival of the immature T2 B cells, which are targets for negative selection, and allows their differentiation to mature B cells [42]. These findings, together with the constitutive expression of BLyS in human leukocytes, indicate that BLyS is a survival factor rather than a true stimulating factor and, thus, it might have a relevant role in the maintenance of B cell homeostasis.

In vivo experiments showed that intraperitoneal administration of the protein caused disruption of the splenic structure with expansion of the white pulp and increased the number of nucleated cells in red pulp [15]. The number of B cells belonging to a terminally differentiated phenotype was increased approximately 10-fold, and circulating IgA and IgM increased two- and five-fold, respectively. BLyS administration enhanced antibody response to T cell-independent antigens, such as Pneumovax, as well as T cell-dependent antigens, such as DNP-BSA [41].

The strongest evidence supporting BLyS as a cytokine regulating the final maturation of the B-2 B cells came from the generation of BLyS null mice [43,44]. In these mice, B-cell development was blocked at the early transitional T1 stage, with extremely reduced numbers of cells of late transitional T2, marginal or follicular phenotypes in the spleen and lymph nodes. The mice had a drastically reduced number of mature recirculating B cells, normal numbers of immature B cells in the bone marrow and of B-1 cells in the peritoneum, and a severely reduced peritoneal B-2 population. The mice had a profound reduction in total serum Ig, but IgA was only slightly inhibited. A reduced antibody response was observed after immunization with T-dependent and T-independent antigens. The number of $CD3^+$ T cells in the periphery was normal, although there was a small reduction in a subset of memory/effector T cells. The other hematopoietic cell lineages were not affected. BLyS deficient mice appeared outwardly normal, with all the major organs and lymphatic tissues present, although the average spleen weight was reduced.

Together, these studies demonstrate that BLyS enhances the magnitude of the humoral immune response by attenuating B cell apoptosis, and suggest that administration of the protein may improve the clinical outcome of patients with immunodeficiencies. The ability of BLyS to stimulate antibody production could be useful in the therapy of Ig deficiencies, such as IgA deficiency, or common variable immunodeficiency disease (CVID), in which B cells fail to differentiate into plasma cells, and consequently very low levels of Ig are present in the sera of patients. BLyS administration may also help to stimulate immune functions in immunosuppressed transplant and cancer patients.

BLYS AS A REGULATOR OF AUTOIMMUNE DISEASES

Multiple components of the immune systems are involved in the pathogenesis of autoimmune diseases. B cells are believed to play a central role because of the production of autoantibodies, for example to nuclear antigens and acetylcholine receptors in patients with systemic *lupus erythaematosus* (SLE) and *myasthenia gravis*. Excessive BLyS-induced survival might disregulate immune tolerance by causing autoreactive B cells to become unresponsive to death signals and to differentiate in antibody-producing plasma cells.

The generation and characterization of BLyS transgenic mice [22,45,46] has highlighted the relationship between

BLyS and autoimmunity. The mice presented enlarged spleens, lymph nodes and Peyer's patches because of the increased numbers of mature B cells and plasma cells. When cultured *in vitro*, the B cells had an activated phenotype and increased survival compared to cells from control mice. In addition, elevated circulating Ig levels were observed in naïve mice. Interestingly, BLyS-transgenic mice developed autoimmune-like phenotypes, with high levels of circulating rheumatoid factor, immune complexes, anti-DNA autoantibodies, and Ig deposition in the kidneys. These pathological changes are similar to the one observed in human SLE that is partially characterized by production of autoantibodies against self antigens, followed by the deposition of immune complexes in the kidney and resulting renal failure.

In accord with these results, high levels of BLyS were found in the sera of NZBWF1 mice, which develop a *lupus*-like syndrome as they age [22]. By 24 weeks of age, the animals had a six-fold increase in serum levels of BLyS compared to controls, and BLyS protein concentration corresponded to disease progression. Further, recent reports have demonstrated increased levels of BLyS in human SLE patients and in patients with other immune-based rheumatic diseases. BLyS levels correlated with serum IgG levels and with anti-double-stranded DNA antibody titers [47,48].

The potential for soluble BLyS receptors and neutralizing anti-BLyS antibodies in the treatment of autoimmune pathologies has been demonstrated by a series of studies conducted in mice. Both IgM and IgG responses to antigen challenge were significantly reduced in mice treated with a recombinant protein consisting of the murine TACI ectodomain fused to the Fc portion of the Ig molecule (TACI-Fc) [25]. In addition, the treatment abolished the formation of splenic germinal centers, which are the sites of antibody affinity maturation and memory B cell formation. Soluble TACI and BCMA inhibited antibody production to both T cell-dependent and -independent antigens and markedly reduced the numbers of peripheral B cells [23–25,49]. Interestingly, in the murine *lupus* model, administration of human soluble receptor fusion protein (TACI-Ig) significantly delayed the frequency of proteinuria and increased the survival of the NZBWF1 animals [22]. Moreover, TACI-Fc injection delayed the onset and lowered the severity of inflammation in a mouse model of collagen-induced arthritis, which resembles human rheumatoid arthritis [34].

The recent identification of a third BLyS receptor, BAFF-R, will conceivably lead to the generation of additional antagonists for the treatment of autoimmune disorders. Moreover, in light of the different specificity of the three receptors, comparison of the effects induced by *in vivo* administration of the decoy receptors will help to define their individual contribution to the regulation of B-cell physiology and humoral immunity.

BLYS AS A TARGETING PROTEIN FOR B CELL MALIGNANCIES

The third and most intriguing part of the therapeutic trichotomy is the possibility that BLyS may find clinical application as a targeting protein for delivery of toxins or radioisotopes to neoplastic B-lineage cells. The rationale for development of a BLyS targeting protein is based on three observations. First, BLyS receptors have been detected on virtually every primary explant of non-Hodgkin's lymphoma (NHL), multiple myeloma (MM) and chronic lymphocytic leukemias (CLL) studied to date ([50], Hilbert D. M. unpublished data). The relative expression of BAFF-R, TACI, and/or BCMA on these tumors has not been reported but the fact that each receptor binds BLyS with a similar affinity suggests that expression of any one BLyS receptor may be sufficient to target labeled BLyS to these tumor types.

Second, several laboratories have reported that the BLyS receptors are not expressed on pro/pre B cells found in the bone marrow. This finding provides the hope that the lack of receptors on B cell precursors will allow a rapid regeneration of the peripheral B cell following labeled BLyS administration. On the other hand, tumors derived from early stages of B cell development such as acute lymphoblastic leukemia (ALL) are not likely targets for a labeled BLyS therapy.

Finally, the plasma half-life of BLyS in mice is a surprisingly long 2.7 h, a value significantly longer than that predicted for other ligands in the TNF superfamily [51–53]. If extrapolated to humans, a labeled BLyS protein would have a predicted plasma half-life of 12–24 h [54], giving the drug ample time to distribute to receptor bearing tumor tissues. Once distributed, the ability of the drug to deliver the cytotoxic signal will depend on the nature of the "label" attached to BLyS and the susceptibility of the tumor to such an insult.

Taken together, BLyS, appears to have the requisite pharmacodynamic and pharmacokinetic properties to be support development of a labeled BLyS protein for the treatment of many neoplastic B-lineage tumors.

CONCLUSIONS

The discovery and biological characterization of BLyS has led to exciting new opportunities in the treatment of B-cell malignancies. At least three approaches can be envisaged in which BLyS-based therapeutics could be employed: recombinant BLyS for the treatment of immune deficiencies; BLyS antagonists for the treatment of autoimmune disorders; and radiolabeled BLyS to treat cancers of the B-cell lineage. Clearly the success of these approaches awaits clinical studies. In addition, it is anticipated that the functional genomic approach employed to discover BLyS, together with the approaches described to exploit the molecule therapeutically, will

herald the discovery of additional proteins useful in treating diseases with unmet medicinal need.

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